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Visualization of cAMP Receptor Protein-induced DNA Kinking by Electron Microscopy

The effect of specific DNA binding of the cAMP · cAMP receptor protein complex to two DNA fragments (301 and 2685 base-pairs in length) containing the *lac* operon has been investigated by electron microscopy. It is shown that specific DNA binding of the cAMP · cAMP receptor protein complex induces a kink of 30 to 45° in the DNA with the apex of the kink located at the site of protein attachment. These findings lend direct visual support for the kinking hypothesis based on the observation of anomalous electrophoretic mobility of DNA fragments containing specifically bound cAMP receptor protein.

The cAMP receptor protein regulates the transcription of at least 15 genes in *Escherichia coli* by binding in the presence of cAMP to specific target sites near each gene it regulates (de Crombrugghe & Pastan, 1978; Ebright, 1982; de Crombrugghe *et al.*, 1984). In some cases, this interaction stimulates transcription (e.g. in the *lac* operon), whereas in others it represses transcription (e.g. the CRP† structural gene). Little is known about the structure of specific CRP · DNA complexes. Supercoil unwinding studies demonstrated that the handedness of the DNA helix is unchanged upon specific binding of CRP (Kolb & Buc, 1982). This was confirmed by circular dichroism studies on CRP complexes with short DNA oligonucleotides comprising specific target sites, which further indicated that CRP induces a conformational change in the DNA, characteristic of a *B* to *C* transition (Fried & Crothers, 1983; Martin *et al.*, 1983). More recently, electrophoretic studies have shown that specific CRP binding significantly reduced the electrophoretic mobility of DNA fragments, 100 to 300 base-pairs in length (Kolb *et al.*, 1983; Crothers & Fried, 1983; Wu & Crothers, 1984). These findings led to the suggestion that CRP binding induces DNA bending (Wu & Crothers, 1984). This proposal is attractive, as modelling studies based on the 2·9 Å resolution crystal structure of the cAMP · CRP complex (McKay & Steitz, 1981) suggest that DNA kinking can potentially increase the number of contacts between CRP and right-handed DNA (Steitz *et al.*, 1983). In order to test the kinking hypothesis, we have carried out an electron microscopy study on specific CRP · DNA complexes. The electron micrographs obtained, lend direct visual support for cAMP · CRP-induced DNA kinking.

The DNA fragments used in these studies were derived from the pUR2 plasmid (Rüther, 1980) and the bacteriophage M13mp2 (Gronenborn & Messing, 1978),

† Abbreviations used: CRP, cAMP receptor protein of *Escherichia coli* (also known as catabolite activator protein or CAP); bp, base-pair(s).

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both of which contain the *lac* operon. The first fragment, 2685 bp in length, was derived by linearizing pUR2 DNA with the restriction enzyme *Pst*I; the second fragment, 301 bp in length, was derived by a partial *Hae*III digestion of M13mp2 DNA. In both cases, the specific target site for CRP is located approximately in the middle of each fragment (see Fig. 1). Samples for electron microscopy were prepared as follows. CRP (10 nM; purified to homogeneity as described by Takahashi *et al.* (1979) from an overproducing *E. coli* strain harbouring the plasmid pBScrp2 (Cossart & Gicquel-Sanzey, 1982)) was incubated for 30 minutes at room temperature with 10 nM (in specific sites) DNA in the presence of 100 μM-cAMP, 40 mM-KCl, 10 mM-Tris (pH 8·0) and 1 mM-EDTA; glutaraldehyde at a final concentration of 0·1% (v/v) was then added to covalently fix bound CRP and the mixture further incubated for a further 30 minutes (Brack, 1981); finally, the sample was dialysed against 40 mM-KCl and 10 mM-Tris·HCl (pH 8·0). Glutaraldehyde fixation was found to be essential in order to avoid complete dissociation of the cAMP·CRP complex from the specific DNA target site during the course of the subsequent manipulations.

For electron microscopic observations, the 301 bp DNA·CRP complex was adsorbed to glow-discharged carbon films for 60 seconds followed by a brief rinse with distilled water and drying from absolute ethanol. Grids were then rotary shadowed with pure tungsten from an angle of 6 to 8° in a modified Leybold-

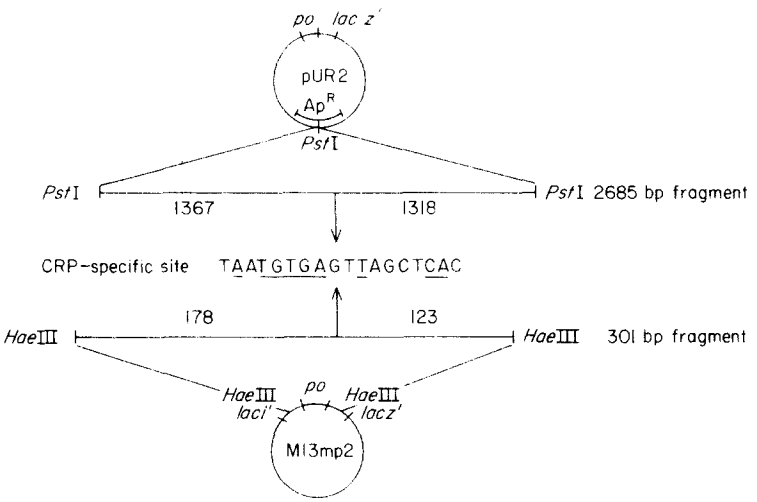

FIG. 1. Schematic representation of the 2 DNA fragments employed in the electron microscopy studies. The 2685 bp *Pst*I fragment consists of linearized pUR2 DNA and was prepared as follows: RR1ΔM15/pUR2 (Rüther, 1980) was grown overnight and plasmid DNA was purified according to Birnboim & Doly (1979). Supercoiled DNA (20 μl at a conen of 1 μg/μl) was digested with *Pst*I, extracted with phenol and precipitated with ethanol. Completion of the linearization was verified by agarose gel electrophoresis. The CRP-specific site of the *lac* operon (Schmitz, 1981) is located approximately in the middle of the fragment as indicated. The underlined bases indicate those that are part of the consensus sequence AA-TGTGA--T---CA-- making up specific CRP target sites (Ebright, 1982). The 301 bp fragment was prepared by partial digestion of M13mp2 DNA (Gronenborn & Messing, 1978), prepared from cleared lysates as described by Clewell & Helinski (1969), with *Hae*III and preparative agarose gel electrophoresis of the resulting fragments. The position of the CRP-specific site is again located approximately in the middle of the fragment. Sequence hyphens have been omitted for clarity.

Heraeus EPA-100 vacuum coating unit fitted with a turbomolecular pump, two electron beam evaporators (Balzers, Leichtenstein) and a quartz-crystal-film thickness monitor (Nanotech, Manchester, U.K.). In some cases, we also employed unidirectional and bidirectional shadowing at an angle of 10°.

Samples containing the 2685 bp long fragment (which corresponds to a length of 912·9 nm for *B* DNA) were mixed with glycerol (7 : 3, v/v) and sprayed on to a freshly cleaved mica surface using a low-pressure air gun (Tyler & Branton, 1980). This approach was superior to simple adsorption or spreading, since less aggregation or collapse of the DNA occurred. The low air pressure also ensured a reduced degree of fragmentation. Electron micrographs were taken with a Philips EM-300 microscope and prints at 72,000× magnification were employed for contour length measurements using a graphics tablet linked to a DEC20/40 computer. The dark field was produced by tilting the electron beam. Instrumental magnification was controlled using catalase crystals.

The free 301 bp DNA fragment appeared under the electron microscope as a straight or slightly curved short filament, adopting a variety of shapes (sigmoid, U-shaped) with no single species predominating. The contour length determined from 150 individual filaments was 103(±12) nm, which is in excellent agreement with the calculated length of 102·3 nm (3·4 Å × 301 bp) for *B* DNA.

Grids of samples containing the 301 bp DNA fragment complexes with cAMP·CRP showed approximately 45% DNA filaments without protein attached and the same percentage of free protein particles. In cases where the protein was bound to the DNA, this was either at one end (86%) or in the middle of the DNA filament (14%; Fig. 2). In most cases (88% out of 50) where the protein was bound in the middle of the DNA, the filament adopted a V shape, with the apex of the V located at the site of protein attachment. Similar V-shaped free DNA was much less frequent (10% out of 500 DNA pieces counted). Since the specific site for CRP is located in the middle of the 301 bp fragment, it is most likely that binding at the ends is due to a non-specific process and only binding at the specific site will be considered for further discussion. The finding of predominantly V-shaped complexes suggests that the cAMP·CRP complex does indeed induce bending or kinking of DNA as has been proposed from electrophoretic studies (Wu & Crothers, 1984). Further inspection of the electron micrographs also reveals that the protein lies on top of the apex of the V with the DNA bent away from the body of the protein.

In order to reduce the non-specific binding to the ends of DNA fragments, we employed a considerably larger DNA piece; namely, the 2685 bp fragment, which also contains the specific CRP binding site in the middle (see Fig. 1). In this case, the number of complexes with CRP bound at the end of the DNA filament was clearly reduced. Of those DNA filaments with protein attached, ~25% contained cAMP·CRP bound in the middle of the ~1 μm filament at the location of the specific target site (Fig. 3(a), (c), (e) and (f)), and ~75% contained cAMP·CRP bound elsewhere (Fig. 3(b) and (d)). This proportion of specific to non-specific binding is as expected on the basis of their respective equilibrium binding constants (Takahashi *et al.*, 1983) taking into account the large excess of non-specific over specific sites (namely, each base-pair can act as the start of a non-

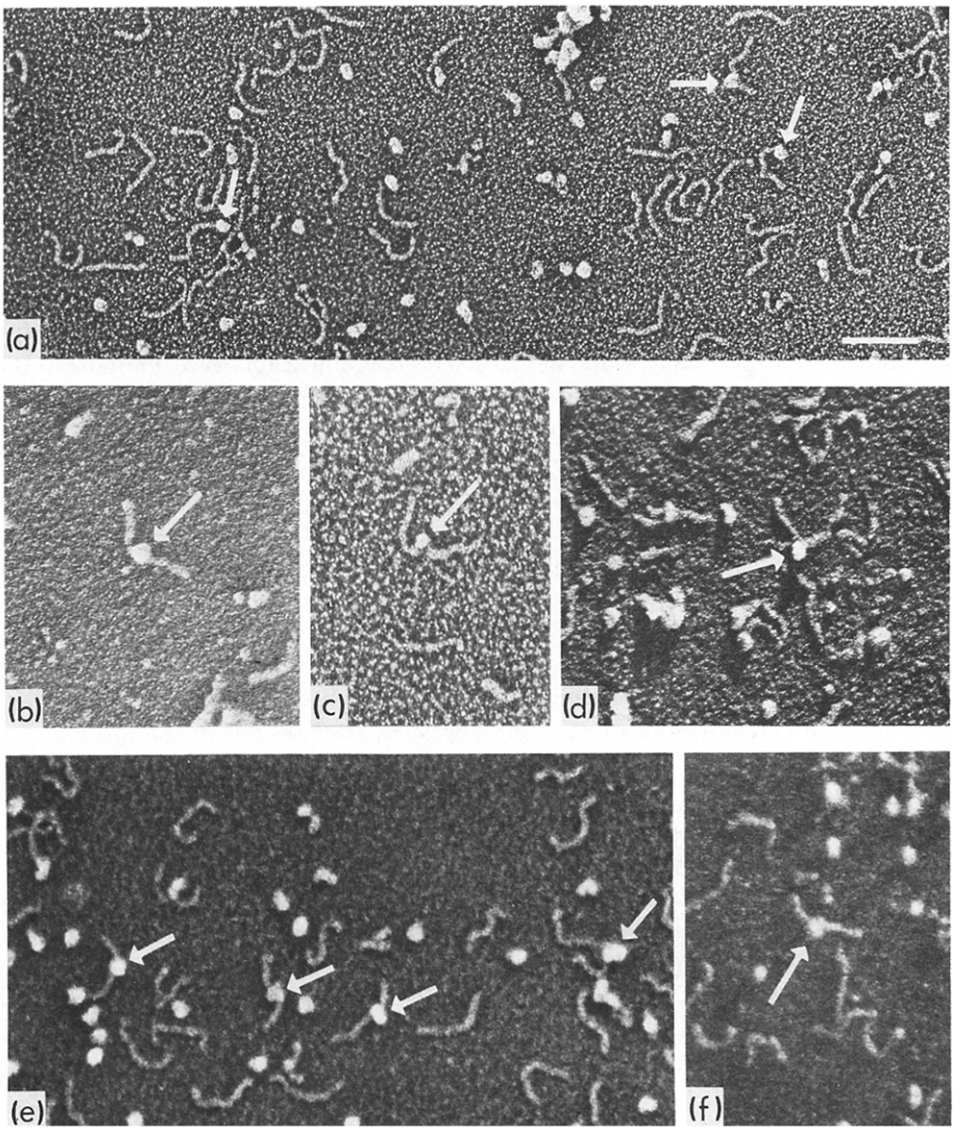

FIG. 2. Electron micrographs of the short (301 bp) DNA fragment and CRP prepared by adsorption to glow-discharged carbon films and rotary ((a), (b) (c) and (e)) or 2-directional shadowing ((d) and (f)). Prints (a) to (d) are reverse copies. (e) and (f) are dark-field images. Magnification in all cases is 100,000×. (a) A general view of the preparation showing free DNA, free protein and DNA·CRP complexes (arrows). (b) to (e) Examples of CRP bound in the middle of the DNA fragment (arrows). The length of the horizontal bar in (a) represents 100 nm.

specific site). Looking at Figure 3, a clearly visible kink is present at the specific target site when the cAMP·CRP complex is bound to it (see particularly Fig. 3(a) and (c)).

Using electron microscopy, we have visualized specific DNA binding of the cAMP·CRP complex associated with kinking of the DNA as well as some non-specific DNA binding. It is most likely that the kink visualized by electron

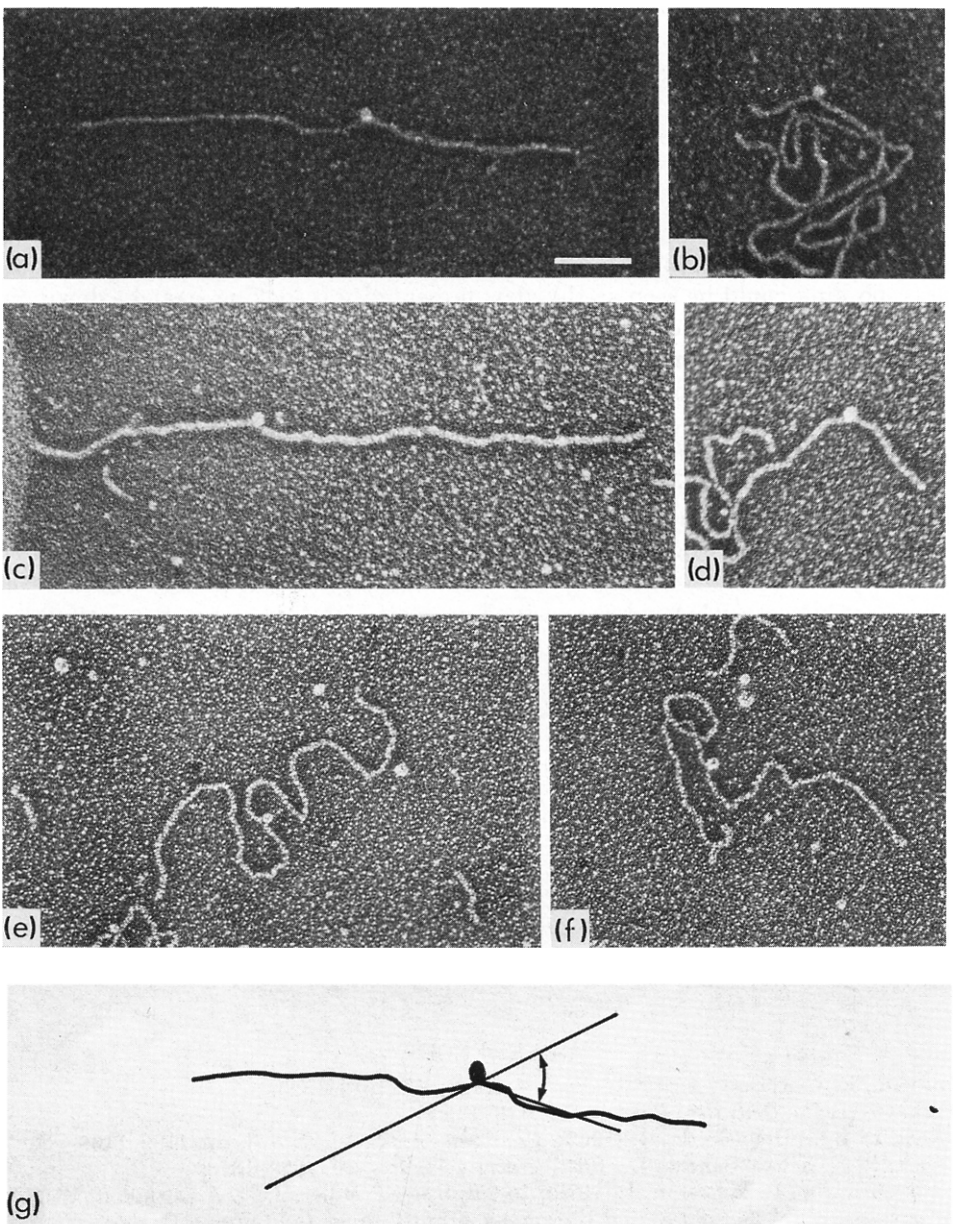

FIG. 3. Electron micrographs of the long (2685 bp) DNA fragment with CRP prepared by spraying with 30% (v/v) glycerol and rotary shadowing with tungsten. (a) and (b) are dark field images, (c) to (f) are reversed copies. The magnification is in all cases 100,000×. (a), (c), (e), and (f) Specific DNA binding of CRP occurs in the middle of the DNA fragment, whereas in (b) and (d) non-specifically bound CRP is located in an eccentric position. Since spraying causes a small degree of fragmentation of the DNA, we measured the length of the longer arm only: in (a), (c), (e), and (g) it was about 0·5 μm, on the average, but in cases of eccentrically located CRP, the ratio of the lengths of the 2 arms of the DNA was about 8 : 2 ((b) and (d)). The diagram in (g) shows how the angle of the kink was measured in the case shown in (a). The length of the horizontal bar in (a) represents 100 nm.

microscopy is responsible for the anomalous electrophoretic mobility of DNA fragments containing specifically bound CRP (Kolb *et al.*, 1983; Crothers & Fried, 1983; Wu & Crothers, 1984). The angle of the kink (Fig. 3(g)), though difficult to measure, appears to vary between 30 and 45°, which is larger than the 12° and 23° bends observed for the neo 1 and 2 kinks, respectively, in the crystal structure of the *Eco*RI endonuclease–oligonucleotide complex (Frederick *et al.*, 1984). This larger kink, however, can be generated easily by a series of smaller in-phase bends. It should also be noted that the apparent variation in the angle of the kink is partly due to the different views present on the supporting film. Indeed, in a few cases, no kink could be seen as the view presented was looking straight down on the top of the kink. Undoubtedly, some of the distortions are due to the surface tension forces acting during air-drying.

The functional significance of the kink is unknown, although it is not difficult to postulate a role for it. For example, kinking may be important in sequence-specific recognition, increasing the number of protein–DNA contacts. This indeed appears to be the case in the crystal structure of the *Eco*RI · DNA complex (Frederick *et al.*, 1984) and has been implicated for the CRP · DNA and Cro · DNA complexes on the basis of model building studies (Steitz *et al.*, 1983; Ohlendorf *et al.*, 1982). In addition, in the case of CRP, kinking may help the formation of complexes between RNA polymerase and promoter DNA, thereby stimulating the initiation of transcription.

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Note added in proof: After submission of this letter a detailed model of the specific complex between CRP and *B* DNA, based on calculations of electrostatic potential energy surfaces, was put forward (Weber, I. T. & Steitz, T. A. (1984) *Proc. Nat. Acad. Sci., U.S.A.* **81**, 3973–3977). In this model the DNA is bent round the body of the protein in contrast to the electron microscopy findings, which clearly show that the DNA is bent away from the body of the protein.